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A Direct Method to Assay Neurotoxic Esterase Activity

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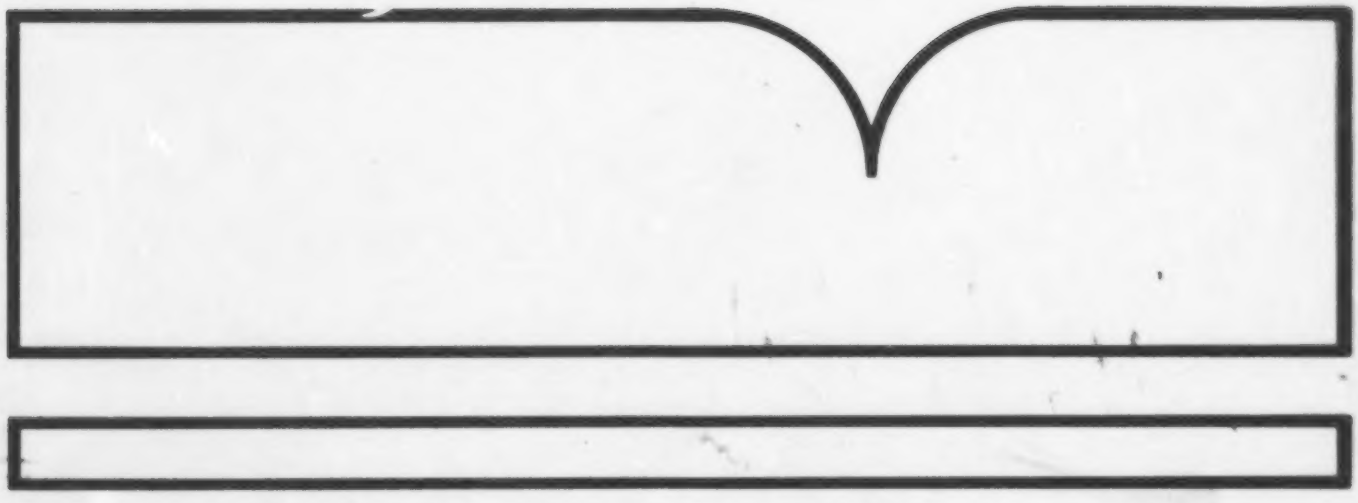

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16. ABSTRACT

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A DIRECT METHOD TO ASSAY NEUROTOXIC ESTERASE ACTIVITY

SALAH A. SOLIMAN, AUGUST CURLEY and ABDEL-KHALEK EL-SEBAE*

*US Environmental Protection Agency, Environmental Toxicology Division, Toxic Effects Branch (MD-67), Research Triangle Park, NC 27711 (U.S.A.), and *Department of Pesticide Chemistry, Faculty of Agriculture, Alexandria University, Alexandria (Egypt)*

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SUMMARY

A direct photometric method for assaying neurotoxic esterase (NTE) activity of chicken brain microsomal preparation has been developed using 4-nitrophenyl esters as substrates. Paired samples of the microsomal preparation were preincubated for 20 min with paraoxon plus either (a) buffer or (b) mipafox before addition of substrate. The initial rate of NTE activity was directly recorded at 410 nm by matching the content of tube (a) against tube (b) after addition of the substrate to both of them. The 4-nitrophenyl esters of propionic, butyric, valeric, lauric, capric and caproic acids were tested as substrates. Results indicated that 4-nitrophenyl valerate and caproate, respectively, are the most hydrolyzable substrates for NTE with this method; it also enables detailed kinetic studies of NTE to be made. The Michaelis constant (K_m) for the hydrolysis of 4-nitrophenyl valerate by NTE was found to be $5.55 \cdot 10^{-5}$ M.

INTRODUCTION

Delayed neurotoxicity is a potential side-effect of some organophosphorus insecticides [1]. Biochemically, it is believed that the target for the delayed neurotoxicants is an esterase present in the nervous tissues. NTE or mipafox-sensitive esterase, is the more sensitive site to be affected preceding the onset of delayed ataxia [2–4]. However, the true physiological substrates and metabolic functions of this enzyme, like the other non-specific esterases, has not yet been clearly defined. It appears that most of the tested delayed neurotoxic organophosphorus compounds or at least one of their metabolites are powerful in vivo or in vitro NTE inhibitors [3–5]. The use of NTE to screen organophosphorus compounds for their ability to induce delayed neurotoxicity is the only biochemical

Abbreviation: NTE, neurotoxic esterase.

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test currently available [4, 6, 7]. Activity of NTE has been assayed with phenyl phenylacetate or phenyl valerate as substrates by a differential assay technique using mipafox as selective inhibitor [3, 6]. Using that technique, paired samples of tissue were preincubated with paraoxon to inhibit paraoxon-sensitive esterases. One tube also contained the neurotoxic organophosphate mipafox which selectively inhibits NTE. Phenyl valerate was then added to both tubes and the phenol produced was determined by blocking the reaction with addition of a sodium dodecyl sulfate solution containing 4-aminoantipyrine. The liberated phenol was determined colorimetrically by conversion into a color complex by the addition of a solution of potassium ferricyanide in presence of 4-aminoantipyrine. Only a terminal figure could be obtained from the material in one pair of the tubes using this technique. Thus, the time-course of the reaction could not be followed without using separate experimental paired tubes for each time interval desired.

Consideration of the relative merits of various methods which might be useful in studying the time-course of activity led to the development of a modified method that converted a substrate enzymatically hydrolyzable into a colored product. Thus, the rate of increasing color intensity during the reaction time-course directly reflected the rate of enzyme activity. This method makes detailed studies of NTE activity possible.

Some 4-nitrophenyl esters are utilized as substrates for NTE. In principle, the method utilizes the direct measurement of the differential rate of production of 4-nitrophenol as the substrate, 4-nitrophenyl ester, is hydrolyzed.

MATERIALS AND METHODS

Chemical grade A, or highly purified grade products (Sigma) were used. A Beckman ultracentrifuge (LS-50), with the appropriate rotors, was used in centrifugation and an Aminco spectrophotometer (DW-2a-UV-VIS) to record the rate of hydrolysis. Mipafox (*N,N'*-diisopropyl phosphoro diamidic fluoride) was synthesized as reported previously [7] while paraoxon was obtained from Aldrich Chemical Company. Brains of adult white leghorn hens were used to prepare the microsomal fraction. In this method, the brain tissue sample was homogenized with 3 vols. of Tris–HCl buffer (50 mM pH 8.0) containing 0.3 M sucrose and 0.2 mM EDTA then the microsomal fraction was isolated by differential centrifugation [7]. The microsomal pellets were resuspended in Tris–HCl buffer (50 mM pH 8.0) containing 0.2 mM EDTA to obtain a suspension of 0.5 mg microsomal protein per 0.1 ml. About 8.0 mg of microsomal protein was obtained from 1 g of wet hen brain tissue. This microsomal fraction has most of the NTE activity [7, 8].

The *p*-nitrophenyl ester solutions were made with dimethyl formamide (33 mg/ml) for the substrate specificity determination or the corresponding equivalent concentration for the kinetic assay. Only 20 μl of any of these should be added to the preincubated microsomal preparation and inhibitor(s) mixture. At this level,

dimethyl formamide does not affect the hydrolyzing activity. The concentration of each substrate was probably above its solubility limit. Mipafox was prepared in Tris–citrate buffer (50 mM, pH 6.0) as solutions from which 200 μl gives concentration of 50 μM, when added to the final enzymic reaction mixture (3.0 ml). Paraoxon was dissolved in acetone and kept as stock. 1 ml of this acetonic solution was diluted with the same Tris–citrate buffer to prepare a solution from which 200 μl gives a concentration of 40 μM when added into the final enzymic reaction mixture. The later solution of paraoxon was prepared freshly each day. The maximum residual amount of acetone has no effect on the enzymic activity. Paired samples of the microsomal preparation (200 μl or the equivalent to the tested protein amount) were preincubated for 20 min with paraoxon plus either (a) buffer or (b) mipafox in a final volume of 2.98 ml using Tris–HCl buffer (50 mM, pH 8.0) containing EDTA (0.2 mM) at 25°C. After preincubation, 20 μl of the substrate solution was added and the initial rate of NTE activity was recorded at 410 nm by matching the content of tube (a) against tube (b) in the spectrophotometer. The

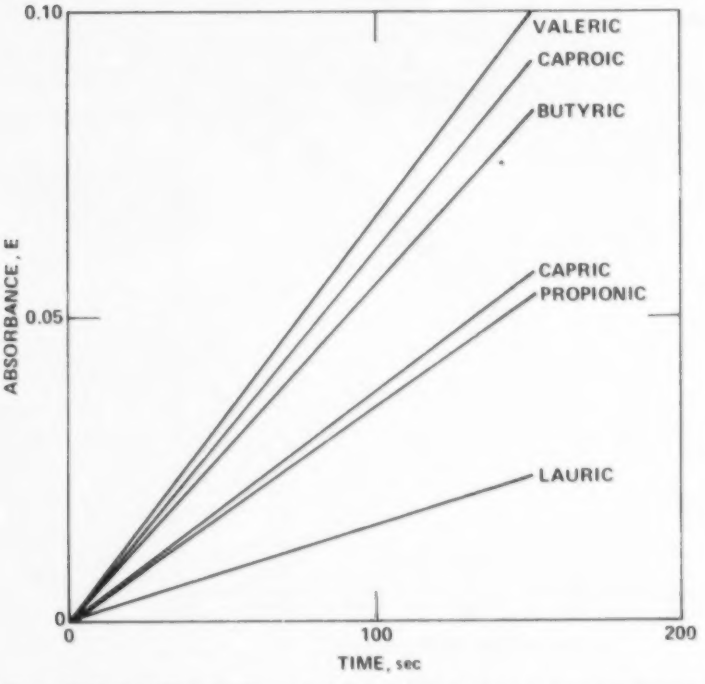

Fig. 1. Hydrolyzability of different 4-nitrophenyl esters of aliphatic acids by chicken brain microsomal NTE. For substrates concentration, see the text.

record reflects directly the NTE activity. The millimolar extinction coefficient, E^{mM}_{410}, of 4-nitrophenol was 11.90 under the conditions of the assay.

Triton X-100, as 1% (w/v) of the stock dimethyl formamidic solution of the 4-nitrophenyl esters, was used to aid dispersal of substrate but this treatment results in relatively high nonenzymatic hydrolysis of the substrate and it has no effect on the enzymic hydrolysis.

An experiment was conducted to compare the new direct method with Johnson's technique. Paired samples of the microsomal preparation (200 μl) were preincubated with the inhibitor(s) as already described. After preincubation, NTE activity was determined by Johnson's method [6] with slight modifications. A 20 μl portion of a dimethyl formamidic solution of phenyl valerate (25 mg/ml) was added to each of the preincubated samples and incubation was continued for an additional 5 min. The enzymic reaction was stopped by the addition of 2 ml of sodium dodecyl sulfate solution (10% w/v in the same buffer but containing 0.025% 4-aminoantipyrine) and the color developed by adding 1 ml of an aqueous solution of potassium ferricyanide (0.4% w/v). The absorbance was determined at 510 nm and the terminal NTE activity calculated [6]. The protein content of the microsomal fraction was determined by the Lowry method [9]. All assays were tested in three or more replicates.

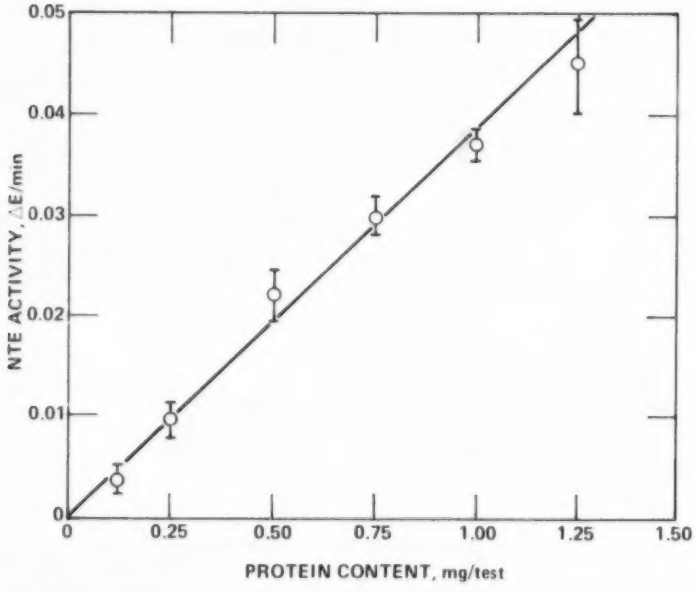

Fig. 2. Effect of microsomal protein content on NTE activity using 4-nitrophenyl valerate as substrate (1 mM). Each point is mean of 3 values ± SD.

RESULTS AND DISCUSSIONS

Fig. 1 represents the time-absorbance curve for the hydrolysis of different 4-nitrophenyl esters by NTE. The rate recorded for the NTE is the net rate because even the non-enzymic hydrolysis rate is automatically avoided in this technique. 4-Nitrophenyl valerate followed by 4-nitrophenyl caproate were the most hydrolyzable substrates by NTE using this method. These results are in agreement with those previously reported for phenyl valerate and caproate as substrates using the Johnson technique [4]. The reaction rates were linear at least for the first 6 min.

The differential rate of hydrolysis of 4-nitrophenyl valerate by NTE as a function of the protein concentration is shown in Fig. 2. The obtained rate was approximately proportional to protein concentration with, perhaps, a small decline in the more turbid samples. The effect of the substrate (4-nitrophenyl valerate) concentration on the NTE activity was estimated. Fig. 3 presents the double reciprocal plot of 4-nitrophenyl valerate concentrations and the corresponding NTE activities. The K_m for the reaction was found to be $5.55 \cdot 10^{-5}$ (Fig. 3). This K_m value is lower than that mentioned by Dr. M.K. Johnson for the hydrolysis of phenyl valerate by NTE using his technique (personal communication). Comparison of the NTE activity of the same chicken brain microsomal preparation using the new method, which is presented here, and Johnson's technique [6] was studied. Results

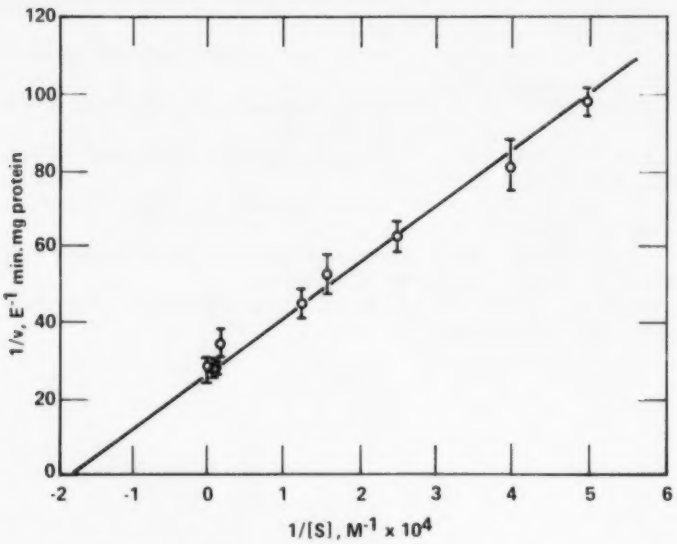

Fig. 3. Double reciprocal plot for the effect of 4-nitrophenyl valerate concentration ([S]) on NTE activity (v).

indicated that the direct method is only one half as sensitive as Johnson's method. The NTE activity of the tested chicken brain microsomal preparation using this new method and the Johnson technique, under the same conditions, was 36 ± 2 (SD) and 72 ± 3 (SD) spectrophotometric units, respectively. One spectrophotometric unit means 0.001 change in absorbance per min/mg protein. We consider that our method has the advantage that the time-course of the NTE activity could be followed directly from the initial time, and thus it makes detailed kinetic studies of NTE activity possible. However, the application of this method to whole homogenate is limited by turbidity.

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